Anticompulsive-like effect of nitric oxide synthase inhibitors in marble-burying test

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Reviewer B:

I thank the editor for the possibility to review the manuscript which I like in many ways. In the end, I think the main conclusion made by the manuscript seems correct and is in agreement with the presented prior work. A slightly more precise description of the attempted replication could still be in order, as the referenced study on NOS inhibitors and marble burying behavior used different NOS inhibitors. I would also like the very concise form of the manuscript if it didn't come together with several problems:

Q. The reporting does not comply with ARRIVE guidelines. This is not mandatory as per journal's guidelines, but is considered the best practice, increasing in importance especially here, where replicability and reproducibility are considered.

R. The present study was conducted in 2010, a few months after the publication of the first article by Krass et al (1). The ARRIVE guidelines were published later that same year, therefore stating that we complied with those guidelines for the study design would be inaccurate. However, we agreed that this guideline is considered best practice and added as much information as possible in the revised version to comply with the essential 10 items for reporting the results.

Q. Sample sizes are not thoroughly described:

a. What was the total number of animals in the study?

R. The total number of animals used was 81 (with 2 excluded due to drug leakage during administration). We now indicate the individual number of animals per group, and state the total number used.

b. How the animals were assigned to the groups?

R. The animals were randomly assigned to the experimental groups by one experimenter, and tested by another experimenter. We now make it clear in the text.

Q. Study design is unclearly reported

a. The vehicle control is not mentioned in the main text but is used as the main comparator

R. The original text stated that ‘All drugs were dissolved in sterile saline…’; we now highlighted that sterile saline was the vehicle.

b. How were the used drug doses chosen?

R. The doses were chosen based on previous studies from our group, as they were cited in the first version of the manuscript: Montezuma et al 2012 (2). We now made it more clear in the main text.

b.1. The doses as mg/kg or µg/kg should be more clearly indicated in the main text, also preferably using either milligrams or micrograms, not both.

R. Given the high dose range among the drugs used, for instance 300mg/kg for AMG and 0.75µg/kg for 1400W, we opted to keep the two different units. Otherwise, we would end with 300.000µg/kg for AMG or 0.00075mg/kg of 1400W.

c. In EPM, for open arms the entries are analyzed as percentages but for enclosed arms as numbers, why?

R. According to the factorial analysis of the elevated plus-maze in mice (3), the percentage of entries and time in the open arms are those most associated with anxiety, while the number of entries in the enclosed arm is the variable most associated with locomotion.

Q. Statistical methods are not mentioned in the main text at all

a. Reasoning for chosen statistical tests is not given.

R. The non-parametric tests were chosen based on the nature of the independent variables. Since 'number of buried marbles' and 'number of entries in the enclosed arm' are discrete measurements, the use of a non-parametric test is recommended . Although the numbers of cases per group in the %OAE and %OAT from the EPM data don't allow the assumption of Gaussian distribution, we did not find any difference in Bartlet's test for homoscedasticity, therefore we conducted one-way ANOVA.

b. Kruskal-Wallis data reported imperfectly (df missing, F-statistics named for ANOVA, but H-statistics not for K-W).

R. The information was added.

c. Info on the used statistical software packages is completely missing.

R. The information was added. Now it reads: 'All data was analyzed using GraphPad Prism (v.5)...'.

Q. While I agree with the referenced idea that marble burying could be considered a repetitive behavior, I question whether a single test run can be called compulsive, and therefore it is questionable if the shown effects are "anticompulsive-like" as the title claims.

R. The main confounding factors in the MBT are locomotor effects of a given compound or classical anxiolytic-like effects such as of diazepines. To control for such false-positive outcomes, it is possible to observe loss of effect upon re-exposure of the animals under repeated drug treatment (4); or lack of anxiolytic properties, such as observed in the present study for AMG. Once these two main points are considered, the term 'anticompulsive-like effect' is widely accepted, similarly to the term

'antidepressant-like effect' for drugs that affect the immobility time in the forced swimming test. Of course, MBT and FST are only screening tests, only fulfilling the pharmacological validity criteria of a model. We believe that a discussion on the validity of the term, as well as the limitations of MBT, are beyond the scope of the present manuscript. Therefore, we opted to keep the term as in the original version.

Q. There is some repetition

The minimization of animal suffering and the related references to relevant protocols is stated twice.

The waiting time of 1h prior the testing is also mentioned twice

R. The repetitions were corrected.

Q. There are inconsistencies

Aminoguanidine being abbreviated AMG and amg.

R. The abbreviations were corrected to AMG

Q. Language need refinement at places

R. The revised manuscript was submitted to the language revision service provided by the University of Helsinki.

Reviewer C:

In this work, the authors have investigated the effects of NOS inhibitors in marble burying behaviour (MBB) and anxiety-like behaviour. They identified that the non-selective NOS inhibitor AMG attenuated MBB, an effect replicated by the selective NOS1 inhibitor, but not NOS2. By assessing the effects of AMG in the elevated plus maze they describe that its effects are specific for MBB, and not anxiety-like behaviour. It is an interesting and compact manuscript, but I have a few concerns listed below.

Q. Why did the authors use the Marble Burying protocol from Njung'e (1991)? There is a more recent protocol from Deacon 2006 (doi:10.1038/nprot.2006.20) that is generally used in recent papers.

R. We opted to use the same protocol from the study of Krass and colleagues (1) we aimed to replicate. The procol from Deacon does not specify the number of marbles, just the distance between them (4cm). Based on the dimensions of the test chamber used in our protocol, so as the one from Krass et al, it reaches close to this recommendation. We, and other groups, have been adapting several parameters of the original studies from Njung'e and Handley (5), and Broekkamp et al (6), changing the number of marbles and time of testing with successful results using positive controls, such as paroxetine (4,7–11). We now included the protocol by Deacon in our references.

Q. Based on which study did the authors choose the drugs doses to be used? Do the authors know whether all three drugs cross the blood-brain barrier?

R. As in the response to a previous question: the doses were chosen based on previous studies from our group, as they were cited in the first version of the manuscript: Montezuma et al 2012 (2). We now made it more clear in the main text. Regarding the brain penetration question, there is evidence that AMG and arginine derivatives are able to enter the brain (12), however through different mechanisms: diffusional process vs saturable basic amino acid transporter, respectively. Moreover, evidence shows that systemically injected 1400W reduced the activity of iNOS in brain lysates (13). Thus we can assume that the behavioral effects observed in the present study may be due to the direct effect of these compounds on NOS isoforms.

Q. Kruskal-Wallis is missing df value.

R. The df values (n of groups - 1) were added to the figure legend.

Q. Two animals were excluded from the NPA 1.3 group. Why is that (it should be declared in the manuscript)?

R. These animals were excluded due to leakage of solution following the intraperitoneal administration, we apologize for not stating it in the first version. However, the addition of these two animals to the group doesn't change the statistical outcomes, from $H(7)=20.78$, $p=0.0041$, to $H(7)=19.55$, $p=0.0066$. The results of the multiple comparisons by Dunn's test also remain.

Q. The sentence about the ethics committee is duplicated.

R. We corrected this mistake.

Q. The manuscript needs an updating in the literature.

R. The literature about the effects of NOS inhibitors or the role of NO in the anticompulsive-like effects of other compounds using the MBT has not been updated recently. The majority of the studies date from late 90's to 2010's, including ours. We unsuccessfully searched the literature for new information in this topic. The most recent original study to our knowledge is Gawali et al 2016 (14), which is cited in the first version of the manuscript, but if the reviewer has a suggestion for a study that escaped our attention, we would gladly consider it.

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